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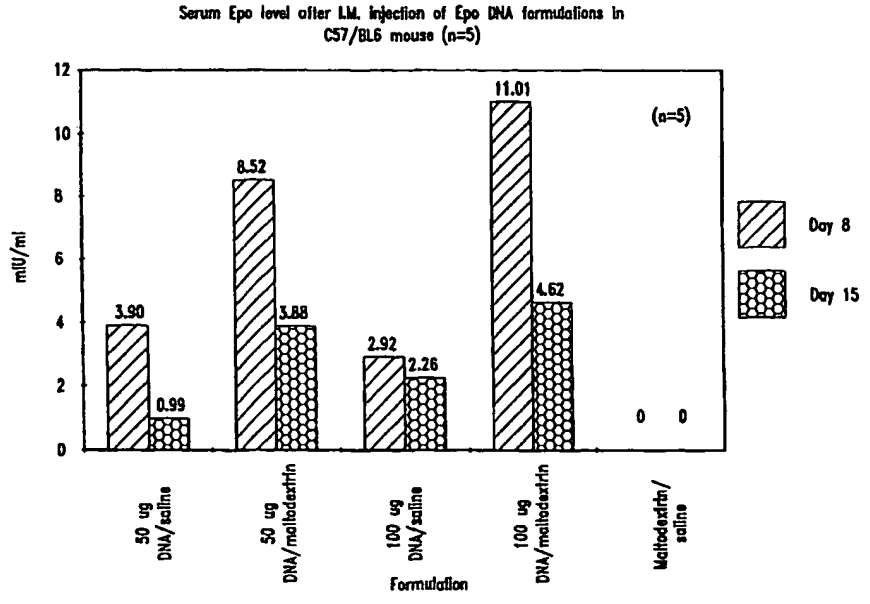

(57) Abstract

Methods and compositions for the delivery of nucleic acids to cells *in vivo* are provided.

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COMPOSITIONS AND METHODS FOR THE DELIVERY OF NUCLEIC ACID MOLECULES

TECHNICAL FIELD

The present invention relates generally to therapeutic compositions and methods, and more specifically, to compositions and methods which are useful for facilitating the uptake of nucleic acids by animal cells.

BACKGROUND OF THE INVENTION

Since the discovery of nucleic acids in the 1940s and continuing through the most recent era of biotechnology, substantial research has been undertaken in order to affect the course of a disease through interaction with the nucleic acids of living organisms. Most recently, a wide variety of methods have been described for altering or affecting genes within humans or animals, by directly administering to the human or animal a nucleic acid molecule which alters or effects the course of a disease. In this regard, many different vectors have been utilized to deliver nucleic acid molecules to a human or animal, including for example, viral vectors derived from retroviruses, adenoviruses, vaccinia viruses, herpes viruses, and adeno-associated viruses (see Jolly, *Cancer Gene Therapy 1*(1):51-64, 1994), as well as direct transfer techniques such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), liposomes of several types (*see, e.g.,* Wang et al., *PNAS 84*:7851-7855, 1987) and administration of nucleic acids alone (U.S. Patent No. 5,589,466 and 5,580,859).

Expression of proteins encoded by nucleic acid molecules following direct intramuscular injection of non-replicating plasmids has been reported (Wolff et al., *Science* 1990, 247, 1465-1468). Such methods may provide a safe and cost-effective treatment for a variety of diseases by producing therapeutic proteins for local or systemic effect (Hartikka et al., *Human Gene Therapy*, 1996, 7, 1205-1217). However, the potential clinical usefulness of direct gene transfer of plasmid DNA (pDNA) in saline can be limited by low levels of gene expression with high variability.

Intramuscular injection of pDNA in saline results in a very small amount of injected pDNA being taken up by cells and expressed, while the majority of the pDNA is rapidly degraded or removed from the muscle (Manthorpe et al., *Human Gene Therapy*, 1993, 4, 419-431). There have been attempts to modify the delivery of plasmids, including, viscosity-increasing polymers such as polyvinylpyrrolidone and polyvinylalcohol (Rolland, A. and Mumper, R.J., WO 96/21470). However, the inclusion of such polymers in large amounts needed to induce favorable responses may be limited by: ease of administration, variability in precise amounts available in the microenvironment to induce biological responses.

The present invention discloses novel compositions and methods for facilitating nucleic acid uptake in cells either *in vivo* or *ex vivo*, and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions for delivering nucleic acid molecules to an animal cell, comprising a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v) neutral or negatively charged polysaccharide, or in a lyophilized composition comprising a neutral or negatively charged polysaccharide. Within certain embodiments, the neutral or negatively charged polysaccharide may be limited to a selected molecular weight (*e.g.*, an average molecular weight of between about 1,000 and about 3,000, 4,000, 5,000, 6,000, 7,000, or, 8,000 daltons one preferred range is between 1,500 and 3,500 daltons), or to a particular species or combination of two or more species of polysaccharides (*e.g.*, mannan, dextran (such as maltodextin), hyaluronic acid, or a glycosaminoglycan such as heparan sulfate, chondroitin sulfate or dermatan). One or more of the polysaccharides may be an adjuvant. Within further embodiments, the compositions provided herein may contain further ingredients or excipients, such as for example, one or more polyalkylene glycols as described herein.

Within other aspects of the invention, compositions and methods are provided for delivering nucleic acid molecules to an animal cell. Within one aspect, compositions are provided comprising a recombinant or synthetic nucleic acid molecule

in an aqueous solution comprising 0.5% to 5% (w/v) polyalkylene glycol, or in a lyophilized composition comprising polyalkylene glycol. Within certain embodiments, the polyalkylene glycol may be limited to a selected molecular weight (*e.g.*, an average molecular weight of between about 1,000 and about 3,000, 4,000, 5,000, 6,000, 7,000, or, 8,000 daltons), or to a particular species or combination of species of polyalkylene glycols (*e.g.*, polypropylene glycol, polyethylene glycol, or, methoxy-polyethylene glycol). Within further embodiments, the compositions provided herein may contain further ingredients, such as for example, a mannan.

Any of the above noted compositions may be utilized to deliver a nucleic acid molecule to an animal cell. Particularly preferred methods for delivering the nucleic acid molecule include intravenous, intramuscular, intraocular, intrapericardial, subcutaneous, intrarticular, intrathecal and mucosal modes of administration. For the treatment of tumors or cancer, the compositions provided herein may be directly administered to a tumor, for example, by stereotatic injection.

A wide variety of nucleic acid molecules may be prepared and/or utilized in the compositions and methods provided herein. For example, the nucleic acid molecule may be a vector that directs the expression of a protein or polypeptide, or which contains an antisense molecule or a ribozyme sequence. Representative examples of proteins or polypeptides which may be encoded by a vector include pro-drug converting enzymes such as thymidine kinase, or, cytosine deaminase, and therapeutic molecules such as, for example, thrombopoietin ("TPO"), erythropoietin, Factor VIII, Factor IX, insulin, growth hormones, leptin and antibodies. Alternatively, the protein or polypeptide may encode an antigen (*e.g.*, from a foreign organism such as a virus, bacteria or parasite, or from a pathogenic cell such as an autoreactive immune cell or a cancer cell), and or, an immunomodulatory co-factor such as IL-2, IL-3, IL-4, IL-10, IL-12, IL-15, or, gamma interferon. Within certain further embodiments, the vector may direct the expression of at least two different recombinant or synthetic nucleic acid molecules (*e.g.*, an immunomodulatory co-factor and a prodrug converting enzyme, or a therapeutic protein or polypeptide such as erythropoietin and a prodrug converting enzyme).

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph which shows reporter gene expression in mouse TA muscle after intramuscular injection of DNA formulations including 2% hyaluronic acid, 2% maltodextrin, 2% PEG (2000), and a saline control (naked DNA).

Figure 2 is a bar graph which shows luciferase reporter gene expression after intramuscular injection of DNA formulations including 2% maltodextrin, and saline (naked DNA control). Data is from pooled results collected using a total of 93 muscles.

Figure 3 is a bar graph which shows CAT activity in mice after intramuscular injection of DNA formulations including 2% maltodextrin, and saline (naked DNA control). Data is from pooled results collected using a total of 20 muscles.

Figure 4 is a bar graph which shows the ELISA results of expression of recombinant mouse erythropoietin *in vivo* by administration using maltodextrin at 75 and 150 ug as compared to DNA/saline controls, after one and two weeks.

Figure 5 is a schematic illustration of luciferase plasmid CMVKM L.UC.

Figure 6 is a graph which shows viscosity measurements of placebo formulations containing varying amounts of maltodextrin.

Figure 7 is a bar graph that shows serum erythropoietin levels after intramuscular injection of EPO DNA formulations in mice.

Figure 8 is a graph that shows the percent hematocrit after intramuscular injection of various amounts of EPO DNA formulations in mice.

Figure 9. Figure 9(a) shows serum EPO levels after intramuscular injection of EPO DNA formulations in SCID mice. Figure 9(b) shows the percent hematocrit of the mice.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Nucleic acid molecule" refers to any deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotide, or nucleic acid fragment generated by polymerase chain reaction, ligation, scission, endonuclease action, or, exonuclease reaction. Nucleic acid molecules can be composed of monomers that are naturally-occurring (*e.g.*, DNA or RNA), or modified monomers (*e.g.*, modifications to sugar portions and/or pyrimidine or purine portions of a nucleic acid). Moreover, the nucleic acids may be linked by phosphodiester bonds, or suitable analogs such as phosphorothioate, phosphorodithioate, phosphoramidate, and the like. Nucleic acid molecules may be single-stranded, double-stranded, or chimeric single- or double-stranded molecules. Nucleic acid molecules may be of any desired length, including for example, as small as 8 to 10 nucleotides, or as large as an entire gene.

"Isolated nucleic acid molecule" refers to a nucleic acid molecule that is not integrated into the genomic DNA of an organism. Examples include both recombinantly and synthetically generated nucleic acid molecules.

A "promoter" is a nucleic acid sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' region of a gene and is proximal to the transcriptional start site of the structural gene.

"Nucleic acid expression vector" or "Vector" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The vector must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within certain embodiments of the invention, the nucleic acid expression vectors described herein may be contained within a plasmid construct. In addition to the components of the nucleic acid expression vector, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning

site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication). Within certain embodiments, the vector may be comprised one or more viral nucleic acids, e.g., a viral promoter and/or envelope sequence.

"Polyalkylene glycols" refer to 2 or 3 carbon polymers of glycols. Two carbon polyalkylenes include polyethylene glycol (PEG) of various molecular weights, and its derivatives, such as polysorbate, polyoxyethylene sorbitan monolaurate, and polyethylene glycol-*p*-isooctylphenyl ether. Three carbon polyalkylenes include polypropylene glycol and its derivatives.

"Polysaccharides" refer to polymers of monomer sugars. These sugars include D-glucose, D-mannose, D-fructose, D-galactose, L-galactose, D-xylose and D-arabinose. In addition, derivative monosaccharides may also be polymerized. Such derivatives include D-glucuronic acid, N-acetyl-muramic acid, and N-acetyl neuraminic acid. Polymers of these monosaccharides may be composed of one type of saccharide or multiple types of saccharides and may be various molecular weights. Naturally occurring polysaccharides that may be used within the scope of this invention include dextrans of various molecular weights (including maltodextrin), a-amylose, amylopectin, amylase-modified versions of polysaccharides, fructans, mannans, xylans, and arabinans.

"Immunogenicity" refers to the ability of a given molecule or a determinant thereof to induce the generation of antibodies upon administration *in vivo*, to induce a cytotoxic response, activate the complement system, allergic reactions, and the like. An immune response may be measured by assays that determine the level of specific antibodies in serum, by assays that determine the presence of a serum component that inactivates the condensing agent/nucleic acid complex or conjugated gene delivery vehicle, or by other assays that measure a specific component or activity of the immune system. Low immunogenicity may be established by these assays. The terms "low immunogenicity," "reduced immunogenicity," "lowered immunogenicity" or similar terms are intended to be equivalent terms.

The present invention provides compositions and methods of delivery of nucleic acids to cells *in vivo*. Briefly, the compositions comprise a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v)

polyalkylene glycol, and/or a 0.5% to 5% (w/v) neutral or negatively charged polysaccharide. Alternatively, the compositions can be provided as a lyophilized composition comprising a polyalkylene glycol or a neutral or negatively charged polysaccharide.

Such compositions provide a greatly increased level of expression of a desired nucleic acid molecule, as compared to compositions wherein the nucleic acid molecule is delivered in saline alone. In addition, the compositions provide a high local concentration of other nucleic acids such as, oligonucleotides, ribozymes, etc., then taken up by cells, they may exert their enhanced biological activities.

PREPARATION OF NUCLEIC ACID MOLECULES

Nucleic acid molecules that can be generated for use within the compositions described herein include both coding and noncoding nucleic acid molecules. Representative examples of suitable molecules include, for example, cytotoxic genes, disease-associated antigens, antisense sequences and ribozyme molecules, sequences which encode gene products that activate a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product, sequences which encode immunogenic portions of disease-associated antigens, replacement genes and sequences which encode immunomodulatory cofactors or immune accessory molecules.

Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen, et al., *J. of Biol. Chem. 266*:6848-6852, 1991: Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987;

Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Antisense molecules are those nucleic acid molecules which are capable of forming a stable duplex or triplex with a mRNA transcript or gene of interest. Antisense molecules can be utilized not only to inhibit or prevent transcription or translation of a desired gene or transcript, but also as a cytotoxic gene in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences may be utilized to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA.

Ribozyme nucleic acid molecules are those molecules which contain a catalytic center. Ribozymes include not only RNA enzymes, but self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions.

Yet other nucleic acid molecules that may be utilized within the context of the present invention include nucleic acid molecules which encode immunogenic portions of disease-associated antigens. As utilized within the context of the present invention, antigens are deemed to be "disease-associated" if they are either associated with rendering a cell (or organism) diseased, or are associated with the disease-state in general but are not required or essential for rendering the cell diseased. In addition, antigens are considered to be "immunogenic" if they are capable, under appropriate conditions, of causing an immune response (either cell-mediated or humoral). Immunogenic "portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

A wide variety of "disease-associated" antigens are contemplated within the scope of the present invention, including for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells. Representative examples of altered cellular components which are normally associated with tumor cells include ras* (wherein "*" is understood to refer to

antigens which have been altered to be non-tumorigenic) and p53*. Other disease-associated antigens which may be encoded by the nucleic acid molecules provided herein include all or portions of various eukaryotic (including for example, parasites), prokaryotic (*e.g.*, bacterial) or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV;" see U.S. Application No. 08/102/132), Human Papilloma Virus ("HPV;" see WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV;" see EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV;" see U.S. Application No. 07/948,358; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV;" U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV;" see U.S. Application No. 07/965,084).

Nucleic acid molecules of the present invention also include "Immunomodulatory cofactors", or, "immune accessory molecules" Briefly, these terms refers to molecules which can either increase or decrease the recognition, presentation or activation of an immune response (either cell-mediated or humoral). Representative examples of immune accessory molecules include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf et al., *J. Immun. 46*:3074, 1991; Gubler et al., *PNAS 88*:4143, 1991; WO 90/05147; EPO 433,827), IL-13 (WO 94/04680), IL-15, GM-CSF, M-CSF-1, G-CSF, CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), CD8, ICAM-1 (Simmons et al., *Nature 331*:624-627, 1988), ICAM-2 (Singer, *Science 255*: 1671, 1992), β-microglobulin (Parnes et al., *PNAS 78*:2253-2 al., *Nature 338*: 521, 1989), LFA3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), HLA Class I, HLA Class II molecules B7 (Freeman et al., *J. Immun. 143*:2714, 1989), and B7-2. Within a preferred embodiment, the heterologous gene encodes gamma-interferon.

Nucleic acid molecules of the present invention also include those sequence which encode proteins that are required to "replace" a normal gene function. As utilized herein, it should be understood that the term "replacement genes" refers to a nucleic acid molecule which encodes a therapeutic protein that is capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect.

Representative examples of such genetic defects include disorders in metabolism, immune regulation, hormonal regulation, and enzymatic or membrane associated structural function. Representative examples of diseases caused by such defects include Cystic Fibrosis (due to a defect in the Cystic Fibrosis Transmembrane Conductance Regulator ("CFTCR"), see Dorin et al., *Nature 326*:614, Parkinson's Disease, Adenosine Deaminase deficiency ("ADA;" Hahma et al., *J. Bact. 173*:3663-3672, 1991), β-globin disorders, Hemophilia A & B (Factor VIII and IX -deficiencies; see Wood et al., *Nature 312*:330, 1984), Gaucher disease, diabetes, forms of gouty arthritis and Lesch-Nylan disease (due to "HPRT" deficiencies; see Jolly et al., *PNAS 80*:477-481, 1983) Duchennes Muscular Dystrophy and Familial Hypercholesterolemia (LDL Receptor mutations; see Yamamoto et al., *Cell 39*:27-38, 1984), and diseases which result in deficiencies of insulin production, or erythropoietin production.

Nucleic acid molecules which encode the above-described sequences may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode immune accessory molecules may be obtained from a depository such as the American Type Culture Collection (ATCC, Manassas, Virginia), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative sources sequences which encode the above-noted immune accessory molecules include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6). It will be evident to one of skill in the art that one may utilize either the

entire sequence of the protein, or an appropriate portion thereof which encodes the biologically active portion of the protein.

Alternatively, known cDNA sequences which encode a desired molecule may be obtained from cells which express or contain such sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989 all of which are incorporated by reference herein in their entirety) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described genes may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

Within preferred aspects of the present invention, the nucleic acid molecules are provided in a composition in the form of a vector which directs the expression of a nucleic acid molecule of interest. Within further embodiments, the vector may direct the expression of more than one nucleic acid molecules. Such multiple sequences may be controlled either by a single promoter, or preferably, by additional secondary promoters (*e.g.*, Internal Ribosome Binding Sites or "IRBS"). Within preferred embodiments of the invention, a gene delivery vehicle directs the expression of heterologous sequences which act synergistically. For example, within one embodiment vectors are provided which direct the expression of a molecule such as IL-12, IL-2, gamma interferon, or other molecule which acts to increase cell-mediated presentation in the T_H1 pathway, along with an immunogenic portion of a disease-associated antigen. In such embodiments, immune presentation and processing of the

disease-associated antigen will be increased due to the presence of the immune accessory molecule.

PREPARATION OF NUCLEIC ACID MOLECULE CONTAINING COMPOSITIONS

As noted above, compositions of the present invention are described that provide a greatly increased level of expression when the composition is administered *in vivo*.

Within one aspect, compositions are provide which comprise a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v) polyalkylene glycol, or in a lyophilized composition comprising polyalkylene glycol. Within certain embodiments, the polyalkylene glycol may be limited to a selected molecular weight (*e.g.*, an average molecular weight of between about 1,000 and about 3,000, 4,000, 5,000, 6,000, 7,000, or, 8,000 daltons), or to a particular species or combination of species of polyalkylene glycols (*e.g.*, polypropylene glycol, polyethylene glycol, or, methoxy-polyethylene glycol).

Within another aspect, the composition comprises a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v) neutral or negatively charged polysaccharide, or in a lyophilized composition comprising a neutral or negatively charged polysaccharide. Within certain embodiments, the neutral or negatively charged polysaccharide may be limited to a selected molecular weight (*e.g.*, an average molecular weight of between about 1,000 and about 3,000, 4,000, 5,000, 6,000, 7,000, or, 8,000 daltons), or to a particular species or combination of species of polysaccharides (*e.g.*, mannan, dextran (such as maltodextin), hyaluronic acid, or a glycosaminoglycan such as heparan sulfate, chondroitin sulfate or dermatan).

Compositions of the present invention can optionally include other or additional pharmaceutically acceptable excipients. Such excipients can be used as fillers, processing aids, other delivery enhancers and modifiers, and the like. Suitable excipients include, for example, calcium phosphate, magnesium stearate, talc, monosaccharides, dissaccharides, polysaccharides, dextrose, low melting waxes, ion exchange resins, and the like, as well as combinations of any two or more thereof. A

thorough discussion of pharmaceutically acceptable excipients is available in "*Remington's Pharmaceutical Sciences*" (Mack Pub. Co., NJ 1991).

Additional agents can also be included in the compositions, such as, for example, marker agents, nutrients, and the like. For example, agents that promote endocytosis of the desired nucleic acids or aid in binding of the nucleic acids to the cell surface, or both, can be incorporated into compositions of the present invention.

Liquid compositions of the present invention can be in the form of a solution, suspension, or emulsion with a liquid carrier. Suitable liquid carriers include, for example, water, saline, pharmaceutically acceptable organic solvent(s), pharmaceutically acceptable oils or fats, mixtures thereof, and the like. The liquid carrier may contain other suitable pharmaceutically acceptable additives, such as solubilizers, emulsifiers, nutrients, buffers, preservatives, suspending agents, stabilizers, and the like. Suitable organic solvents include, for example, monohydric alcohols, such as ethanol, and polyhydric alcohols, such as glycols. Suitable oils include, for example, soybean oil, coconut oil, olive oil, safflower oil, cottonseed oil, and the like. For parenteral administration, the carrier can also be an oily ester such as ethyl oleate, isopropyl myristate, and the like.

METHODS OF UTILIZING THE COMPOSITIONS PROVIDED ABOVE

As noted above, the present invention provides methods for delivering nucleic acid molecules to an animal, comprising the general step of administering to an animal one of the nucleic acid molecule containing compositions described herein. Utilizing such methods, one can greatly enhance the uptake and *in vivo* expression of a desired nucleic acid molecule.

Direct administration to an animal or group of cells within an animal can typically be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to an organ or tissue or to the interstitial space of an organ or tissue. The compositions can also be administered into a tumor or lesion. Other modes of administration include oral and pulmonary administration, rectal or vaginal suppositories or creams, and transdermal applications, needles, and

gene guns or hyposprays, including nasal, oral or other mucosal sprays. Administration can also be by inplants, pumps, catheters or patches.

Modes of administration include, for example, *ex vivo* administration to samples derived from an animal and *in vitro* administration to the sample. The sample can then be readministered to the animal after the cells have contacted and taken up the nucleic acid.

As utilized herein, the term animal includes all living organisms including eukaryotes and prokaryotes, vertebrates and invertebrates, reptiles, birds, and mammals, and within the category of mammals, includes rodents and humans among other subcategories. The compositions of the invention can be administered to any animal or to a group of cells or to a tissue from an animal or in an animal.

Representative examples of tissues to which the compositions of the present invention may be delivered include tissue from mammals, such as, muscle, skin, brain, lung, liver, spleen, blood, bone marrow, thymus, heart, lymph, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, connective, and the like. The target tissue for the administration will be selected based on the nucleic acid to be taken up by the cells of the tissue and the goals of the therapy or administration.

Cells suitable for use in the practice of the present invention include any cell inside an organism or animal, including mammals, particularly humans. Cell lines can also be used for the invention, for example, mammalian cell lines available from the American Type Culture Collection (ATCC), Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), human embryonic kidney cells, baby hamster kidney cells, mouse sertoli cells, canine kidney cells, buffalo rat liver cells, human lung cells, human liver cells, mouse mammary tumor cells, and other mammalian (including human) cells (*e.g.*, stem cells, particularly hemopoietic cells, lymphocytes, macrophages, dendritic cells, tumor cells and the like).

Compositions of the present invention can be administered in order to treat a particular condition or disease. In this case, the composition is administered in a therapeutically effective amount. As utilized herein, a "therapeutically effective

amount" should be understood to refer to those amounts which result in a physiologically significant effect. An effective dosage of nucleic acid administered directly to a subject will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular nucleic acid and encoded polypeptide, the mode and route of administration; the age, health, and weight of the recipient; nature and extent of symptoms; kind(s) of concurrent treatment, frequency of administration, the effect desired, and the like. However, the precise amount for a particular patient and nucleic acid or polypeptide can be readily determined by routine experimentation by a clinician of ordinary skill in the art. Compositions of excipients and nucleic acids can be administered as a single dose or in multiple doses. Multiple doses can be administered either continuously, in intervals, or a combination of both. For purposes of the present invention, an effective *in vivo* amount of nucleic acid will be from about 0.01 mg/kg to about 50 mg/kg or about 0.05 mg/kg to about 10 mg/kg of nucleic acid.

Enhanced nucleic acid uptake by cells can be detected by using protein expression assays (where the nucleic acid encodes a polypeptide) or polynucleotide hybridization techniques (for either coding or noncoding nucleic acids). Compositions can be screened and optimized with respect to transfection efficiency by incorporating a reporter gene into the DNA and assaying for the reporter gene product using standard immunoassay methods or biological or enzymatic activity assays (such as, for example, a luciferase assay).

The concentration of nucleic acid in stable preparations of excipient and nucleic acid is greater than the nucleic acid concentration of the dilute nucleic acid solution, and is typically at least about 150 μg (polynucleotide)/ml (preparation). More typically, the concentration of polynucleotide in stable preparation is at least about 250 μg/ml, more typically at least about 500 μg/ml, and even more typically at least about 1 mg/ml, and even more typically at least about 2 mg/ml.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF COMPOSITIONS OF pDNA AND EXCIPIENTS

DNA plasmids containing a cytomegalovirus (CMV) promoter and chloramphenicol acetyltransferase (CMV-km-CAT), luciferase (CMV-km-Luc) or, β-galactosidase (CMV-km- or β-gal) reporter genes were prepared and purified. Detailed sequences of the plasmid constructs such as shown in Figure 5 are provided in co-pending application entitled "Lipid-conjugated polyamide compounds", U.S. Provisional Application No. 60/054,743.

Maltodextrin (dextrose equivalent 13.0-17.0) and alginic acid (sodium salt) were obtained from Aldrich (Milwaukee, Wisconsin). Hyaluronic acid (from bovine trachea, sodium salt), chondroitin sulfate A, and mannan were from Sigma (St. Louis, Missouri). Polyethylene glycol (2000) was from Fluka (Ronkonkoma, New York). CAT ELISA kit was purchased from Boehringer Mannheim (Indianapolis, Indiana). Luciferase reagent was obtained from Promega (Madison, Wisconsin). The excipients were made in 2% w/v except for alginic acid which was prepared in a 0.5% w/v solution. A series of pharmaceutically allowed excipients in formulations containing plasmid DNA were screened to identify an optimal DNA delivery for intramuscular applications.

All the formulations were prepared fresh on the day of injection. Formulations were made by aliquoting appropriate volumes of stock solutions of pDNA, 5M NaCl, and excipient stock solution to obtain a final pDNA concentration of 1 ug/ul in 150 mM saline with different excipient levels. Viscosities of formulations containing different levels of maltodextrin were monitored using Brookfield viscometer and all formulations used herein had viscosities comparable water for injection (see Figure 6).

Five to six week old female Balb-c mice (20-25 grams, from Charles River) were used in all the animal studies. The mice were anesthetized by intraperitoneal administration of Ketamine/Xylazine cocktail. Plasmid DNA (25 or 50

ug) in 50 ul of the formulation was injected into the tibialis muscle of both legs after shaving the hair around the muscle. Injection depth was limited to 2 mm by inserting a plastic collar to the needle. Naked DNA injections in saline (plasmid DNA + saline) served as controls. Group sizes ranged from 3 to 10. In cases were large animal numbers were used to assess the variations in the data, pooled information from different experiments was used.

Animals were sacrificed 8 days post-injection and the TA muscles were harvested and stored at -70°C until assay for luciferase activity or chloramphenical acetyltransferase (CAT). Frozen TA muscles were individually pulverized into fine powder with a dry ice-chilled porcelain mortar and pestle. Frozen powders were then extracted using lysis buffer. Expressed proteins such as luciferase or CAT were extracted from the muscle with lysis buffer (Promega) followed by centrifugation at 13,6000g for 5 minutes at room temperature. Luciferase activity was assayed using an automated Dynatech model ML2250 microplate luminometer. The amount of CAT protein was assayed by CAT ELISA following manufacturer's instructions.

Results are shown in Figures 1, 2 and 3. Briefly, enhanced luciferase gene expression was observed in initial *in vivo* experiments (Figure 1) using plasmid DNA encoding luciferase gene injected into mouse TA muscles with 2% PEG 2000, 2% maltodextrin or 2% hyaluronic acid. Results shown in Figure 1 are pooled information obtained from DNA injections in 13 muscles. Direct injection of a plasmid DNA (50 ug in saline) into mouse TA muscle produced an average of 4.9 ng of luciferase per muscle at 8 days post injection. Injection of DNA formulations containing PEG 2000 or maltodextrin (2% w/v) resulted in 30–fold enhancement of luciferase expression; hyaluronic acid formulation also showed a 20-fold enhancement in gene expression. The overall result obtained by IM injections in 93 muscles using maltodextrin formulations showed a 5-fold enhancement of luciferase activity compared to injections of naked DNA in saline (Figure 2). This result was further confirmed by injecting CAT (chloramphenicol acetyl transferase) reporter gene. The injections of CAT reporter gene using 2% (w/v) of maltodextrin has been studied. A 10–fold enhancement in the level of CAT gene expression with formulations containing 2% (w/v) of maltodextrin over saline formulation of DNA was observed (Figure 3). Immunohistochemical

staining of β-gal in muscle also demonstrated that pDNA formulation in 2% polyethylene glycol 2000 was better dispersed in muscle tissue compared to pDNA injected in saline. The staining also showed that the PEG formulation resulted in an increase in the number of cells expressing β-gal and that these cells were distributed over a larger area compared to pDNA injected in saline (data not shown).

EXAMPLE 2

EPO ADMINISTRATION WITH MALTODEXTRIN

A. Preparation of DNA formulations

All the formulations were prepared fresh on the day of injection. Formulations were made by aliquoting appropriate volumes of stock solutions in the following order: water, 5M NaCl, excipient, and pDNA to obtain a final pDNA concentration of 1.5 μg/μl or 0.75 μg/μl in 150 mM saline with 2% Maltodextrin.

B. Injection of Animals

C57/BL6 mice were anesthetized by intraperitoneal administration of Ketamine/Xylazine cocktail. Baseline hematocrit were measured before injection of DNA formulation. Plasmid DNA (see U.S. Application No. 08/910,647, filed August 13, 1997) in 50 μl of the formulation was injected into the tibialis muscle of both legs. Injection depth was limited to 2 mm by inserting a plastic collar to the needle.

C. Measurement of serum monkey EPO concentration

After measurement of the hematocrit, serum was recovered from the capillary tubes and stored at -20C for the measurement of EPO concentration. Serum concentrations of monkey EPO were determined by ELISA system (Quantikine IVD, R&D systems, Minneapolis, MN) using a mouse monoclonal antibody according to the manufacturer's protocol.

D. Results

Results are shown in Figure 4. Briefly, while DNA in saline (at concentrations of 75 or 150ug) do show expression after 1 or 2 weeks, administration of DNA in maltodextin shows an increase of at least 2 to 4-fold in the quantity of EPO expressed.

EXAMPLE 3

INTRAMUSCULAR DELIVERY OF PLASMID DNA FORMULATIONS ENCODING ERYTHROPOIETIN

According to this example, a single intramuscular injection of plasmid DNA encoding EPO in a 2% (w/v) maltodextrin formulation elevated the hematocrit to at least 10% higher level compared to DNA administration in saline. A dose response study showed that mice injected with 50 μg DNA in maltodextrin formulation can reach the same hematocrit levels as mice injected with 100 μg DNA in saline formulation.

DNA plasmids containing a CMV promoter and EPO gene (CMV-EPO) were prepared as described above. Maltodextrin (dextrose equivalent 13.0-17.0) was obtained from Aldrich (Milwaukee, WI). EPO Elisa kit was purchased from R&D systems (Minneapolis, MN).

A. Preparation of DNA formulations

All the formulations were prepared fresh on the day of injection. Formulations were made by aliquoting appropriate volumes of stock solutions in the following order: water, 5M NaCl, 10% maltodextrin, and pDNA to obtain a final pDNA concentration of 0.2 μg/μl ~ 2 μg/μl in 150 mM saline with or without 2% (w/v) maltodextrin.

Example: Preparation of 350 μl of DNA formulation in 2% (w/v) maltodextrin at 1 mg/ml concentration.

H_2O	190.5 µl
10% maltodextrin stock	70 µl
5 M NaCl	10.5 µl
DNA stock 4.42 mg/ml	79 µl
Total volume	350 µl

B. Intramuscular administration of plasmid DNA formulations to mice

Balb/c, C57/BL6 or SCID Beige mice were used in the experiments. The mice were anesthetized by intraperitoneal administration of Ketamine/Xylazine cocktail. Plasmid DNA (10- 100 µg) in 50 µl of the formulation was injected into the tibialis muscle of both legs. Injection depth was limited to 2 mm by inserting a plastic collar to the needle. Hematocrits were determined periodically by centrifugation of blood in a micro-capillary tube. Serum EPO levels were assayed by ELISA using the human EPO Quantikine IVD kit from R & D systems according to manufacturer's recommendations.

Four different experiments were performed:

Experiment	Mouse strain	N=	Dose (µg)	Time points
JM 86	C57/BL6	5	75, 150	weekly for 2 weeks
CH040	Balb/c	5	10, 50, 150	weekly for 5 weeks
CH048	C57/BL6	8	25, 50, 100	weekly for 8 weeks
CH051	SCID-Beige	5	150	weekly for 10 weeks

C. Intramuscular injection of monkey EPO DNA in 2% maltodextrin formulation produced higher levels of EPO in the systemic circulation of immunocomponent mice compared to injection of DNA in saline formulation. (Figure 7)

To determine whether intramuscular injection of plasmid DNA encoding monkey EPO in maltodextrin formulation could produce higher EPO levels in serum compared to DNA in saline, two doses of DNA (50 µg and 100 µg) in either saline or in 2% maltodextrin formulations were injected in C57/BL6 mice. The control group received 2% maltodextrin in saline containing no DNA. Monkey EPO levels in mouse

serum were determined by ELISA at week one and week two after injections using the human EPO Quantikine IVD kit. Results shown in Figure 7 demonstrate the time course of EPO secretion and dose response. Animals injected with either 50 μg or 100 μg EPO DNA formulations containing 2% maltodextrin showed higher serum EPO levels compared to animals that received DNA in saline. Control animals (maltodextrin in saline) had undetectable levels of monkey EPO . Even though EPO levels declined with time, the maltodextrin formulation groups still maintained two- to four-fold higher EPO levels in serum than the DNA/saline group.

D. Dose-response study of the amount of DNA injected and the elevation in hematocrit. (Figure 8)

Biological activity of the recombinant EPO is shown by the elevation of hematocrit in the experimental animals. C57/BL6 mice were injected intramuscular with 25, 50 or 100 μg of EPO plasmid in formulations containing either 2% maltodextrin or saline. Maltodextrin in saline served as buffer control. Hematocrits were monitored periodically during the 60 days after injection. Preinjection values of hematocrit were around 48%. Injections of control buffer alone did not result in any elevation of the hematocrit levels. Injections of low dose formulations (25 μg) elevated hematocrit levels very minimally. However, injections of 100 μg of DNA in maltodextrin formulations resulted in significant elevations in hematocrits to levels of 62 +/- 1.5% at 21 days post injection. The hematocrits of comparable animals which received 100 μg DNA in saline were in the range of 54 +/- 1.6%.

Thus, the observed levels of hematocrit were not only proportional to the amounts of EPO DNA injected but also affected by the DNA formulations. Compared to control animal groups, the hematocrits of animals that received 100 μg DNA in maltodextrin formulations remained elevated for up to 4 weeks, then declined to baseline levels. Second injection did not elevate the hematocrit levels further, probably due to immune responses to monkey EPO expression in mice. Similar experiments were performed in Balb/c mice, and the hematocrits remained high for 2 weeks (data not shown). This observation demonstrates the relevance of transgene expression in homologous animals.

E. Hematocrit levels remain elevated for longer periods after intramuscular injection of DNA formulations in immunodeficient mice (Figure 9)

To determine whether intramuscular administration of DNA formulations could produce sustained expression of monkey EPO in immunodeficient mice, SCID (severe combined immunodeficiency) beige mice were injected intramuscularly with 150 μg of monkey EPO plasmid in maltodextrin formulation. The serum EPO levels declined from 14 mIU/ml at one week post injection to undetectable levels at week five (Figure 9a). Hematocrits of the DNA-injected mice rose from preinjection values of 43% to values of 65% within 3 weeks of injection and remained elevated at (or above) this level for 10 weeks during the course of the experiment. These elevated hematocrits were significantly different from those of control mice injected with buffer control. (Figure 9b)

This example demonstrates that a single intramuscular injection of EPO plasmid in 2% maltodextrin formulation resulted in significant elevation in hematocrits of mice compared to administration of DNA in saline. A dose relationship between the amount of DNA injected and the subsequent elevation in hematocrit has also been demonstrated. Results demonstrate a long-term EPO expression in immunodeficient mice after single intramuscular injection of plasmid DNA encoding monkey EPO cDNA.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. A composition for delivering nucleic acid molecules to an animal cell, comprising a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v) neutral or negatively charged polysaccharide, wherein said polysaccharide has an average molecular weight of between 1,000 and 8,000 daltons.

2. The composition according to claim 1 wherein said polysaccharide is maltodextrin.

3. The composition according to claim 1 wherein said nucleic acid molecule is an antisense molecule or a ribozyme.

4. The composition according to claim 1 wherein said nucleic acid molecule encodes a polypeptide.

5. The composition according to claim 1 wherein said polypeptide is a biologically active molecule selected from the group consisting of TPO, erythropoietin, Factor VIII, Factor IX, and leptin.

6. The composition according to claim 1 wherein said composition comprises at least two different recombinant or synthetic nucleic acid molecules.

7. The composition according to claim 1 wherein said nucleic acid molecule encodes two different polypeptides.

8. The composition according to claim 1 wherein said nucleic acid molecule is an expression vector.

9. A composition for delivering nucleic acid molecules to an animal cell, comprising a recombinant or synthetic nucleic acid molecule in an aqueous solution comprising 0.5% to 5% (w/v) polyalkylene glycol, wherein said polyalkylene glycol has an average molecular weight of between 1,000 and 8,000 daltons.

10. The composition of claim 9 wherein said nucleic acid molecule encodes a polypeptide.

11. The composition according to claim 10 wherein said polypeptide is a biologically active molecule selected from the group consisting of TPO, erythropoietin, Factor VIII, Factor IX, and leptin.

12. A method for delivering a nucleic acid molecule to an animal, comprising administering to an animal a composition comprising aqueous solution comprising 0.5% to 5% (w/v) polyalkylene glycol, wherein said polyalkylene glycol has an average molecular weight of between 1,000 and 8,000 daltons.

13. A method for delivering a nucleic acid molecule to an animal, comprising administering to an animal a composition comprising a recombinant or synthetic nucleic acid molecule in a lyophilized composition comprising polyalkylene glycol, wherein said polyalkylene glycol has an average molecular weight of between 1,000 and 8,000 daltons.

14. A method for delivering a nucleic acid molecule to an animal, comprising administering to an animal a composition comprising an aqueous solution comprising 0.5% to 5% (w/v) neutral or negatively charged polysaccharide, wherein said polysaccharide has an average molecular weight of between 1,000 and 8,000 daltons.

15. A method for delivering a nucleic acid molecule to an animal, comprising administering to an animal a lyophilized composition comprising a neutral or

negatively charged polysaccharide, wherein said polysaccharide has an average molecular weight of between 1,000 and 8,000 daltons.

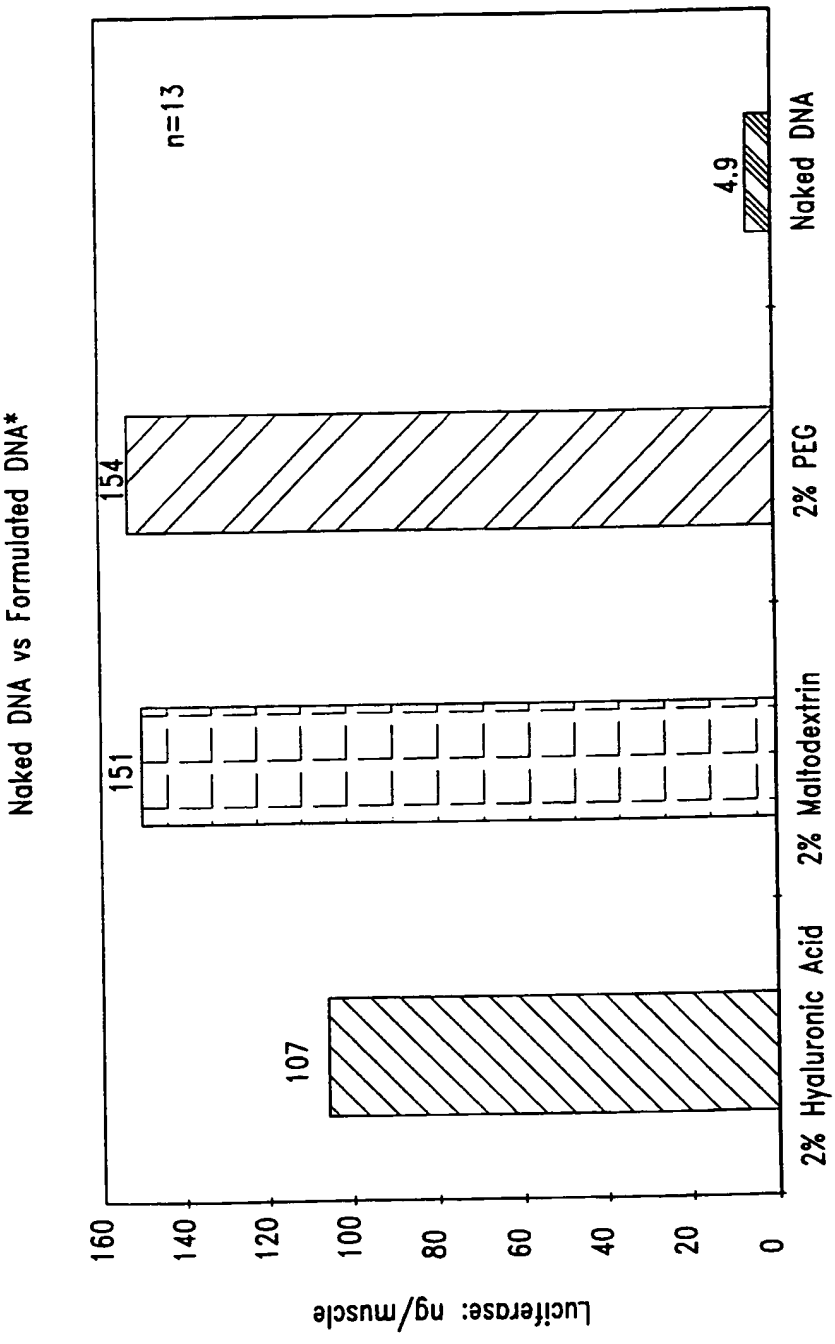
16. The method according to claim 14 wherein said polysaccharide is maltodextrin.

17. The method according to claim 15 wherein said polysaccharide is maltodextrin.

18. The method according to claim 14 wherein said nucleic acid molecule encodes a polypeptide, wherein said polypeptide is a biologically active molecule selected from the group consisting of TPO, erythropoietin, Factor VIII, Factor IX, and leptin.

19. The method according to claim 14 wherein said nucleic acid molecule encodes a polypeptide wherein said polypeptide is a protein selected from the group consisting of a growth factor, an antigen, and an antibody.

20. The method according to claim 14 wherein said nucleic acid molecule is an expression vector.



*50 µg luciferase DNA was injected into mouse TA muscle, luciferase activity was measured one week postinjection

Fig. 1

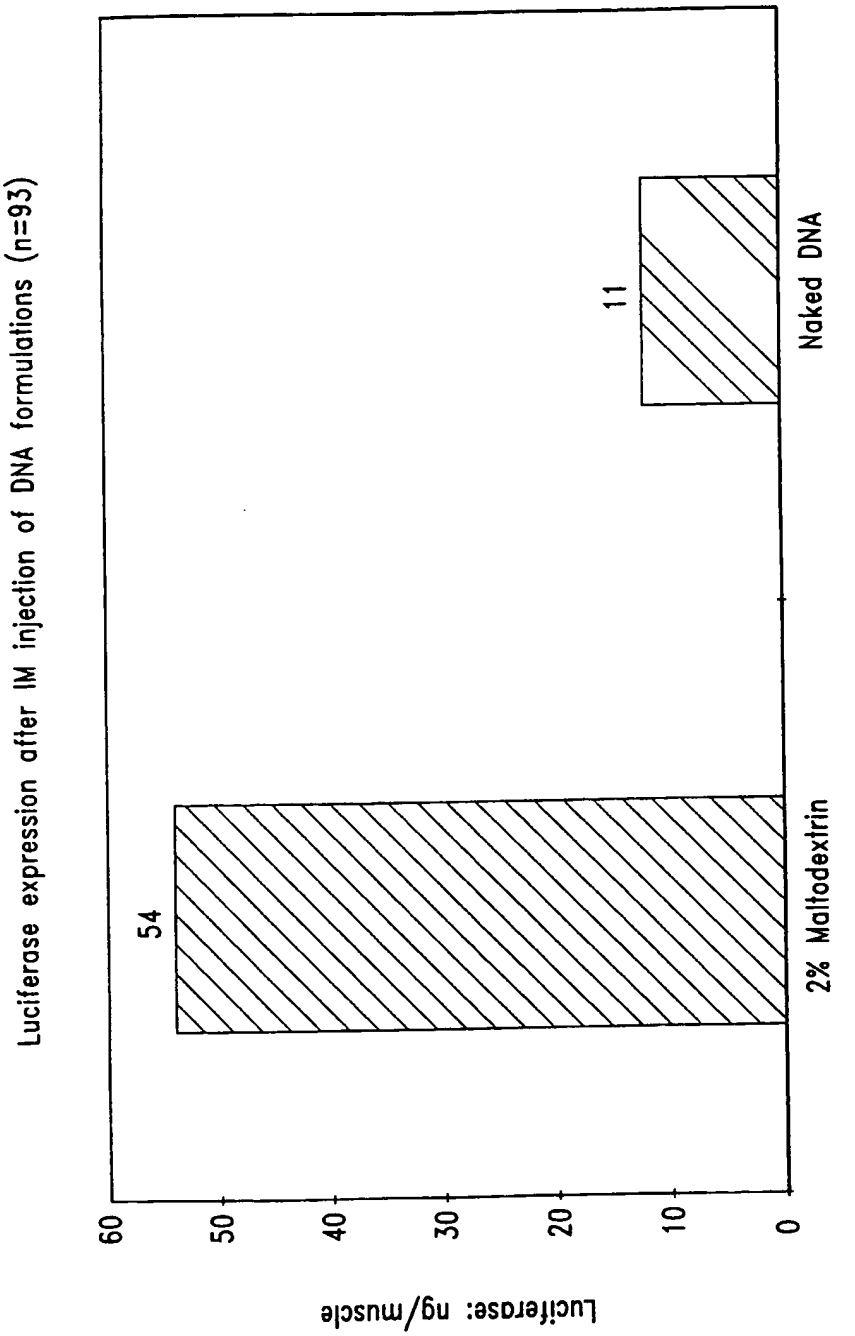

Luciferase expression after IM injection of DNA formulations (n=93)
Luciferase: ng/muscle
60
50
40
30
20
10
0
54
11
2% Maltodextrin
Naked DNA
Formulation

Fig. 2

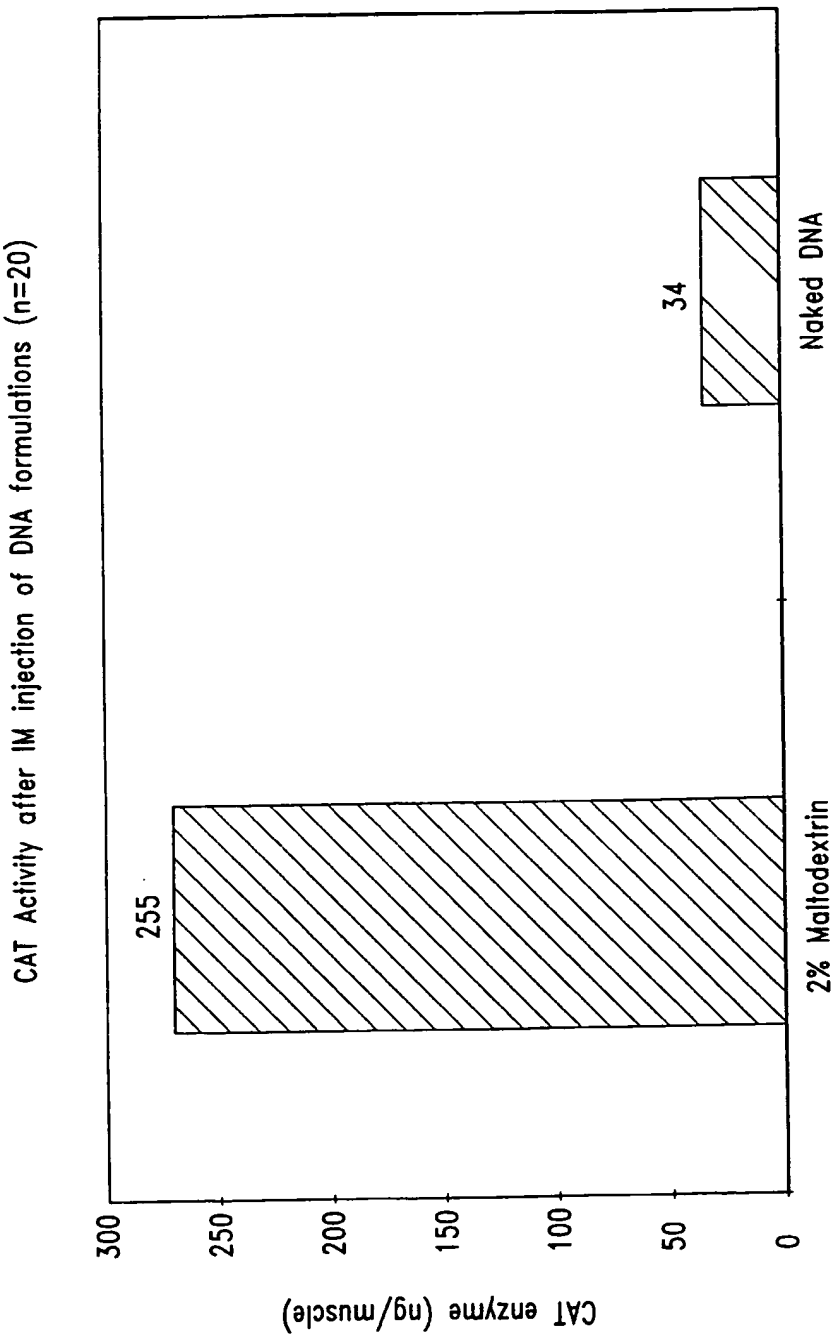

CAT Activity after IM injection of DNA formulations (n=20)
CAT enzyme (ng/muscle)
300
250
200
150
100
50
0
255
34
2% Maltodextrin
Naked DNA
Formulation

Fig. 3

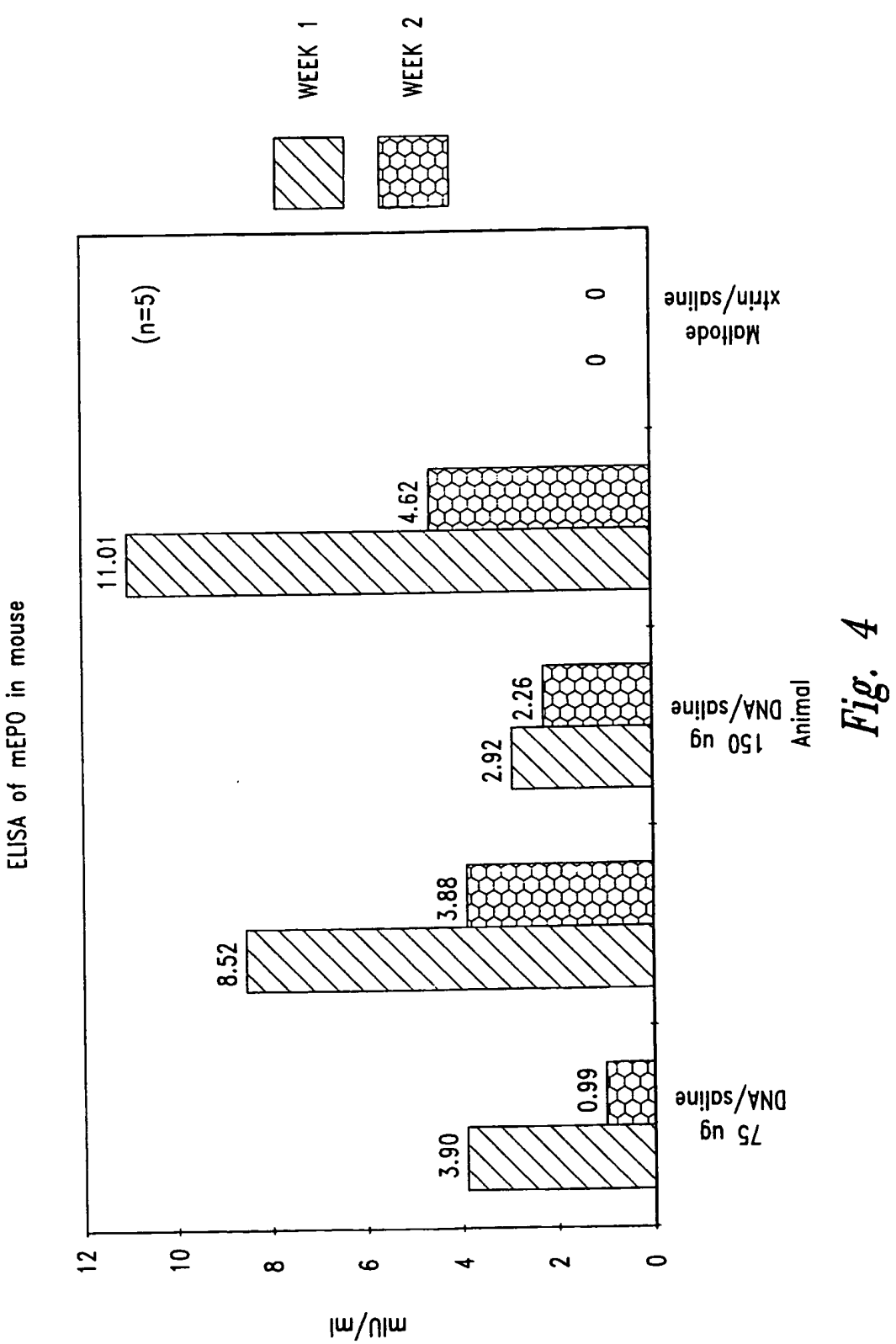

WEEK 1
WEEK 2
ELISA of mEPO in mouse
mIU/ml
12
10
8
6
4
2
0
(n=5)
3.90
0.99
8.52
3.88
2.92
2.26
11.01
4.62
0
0
75 ug DNA/saline
150 ug DNA/saline
Mallotoxin/saline
Animal

Fig. 4

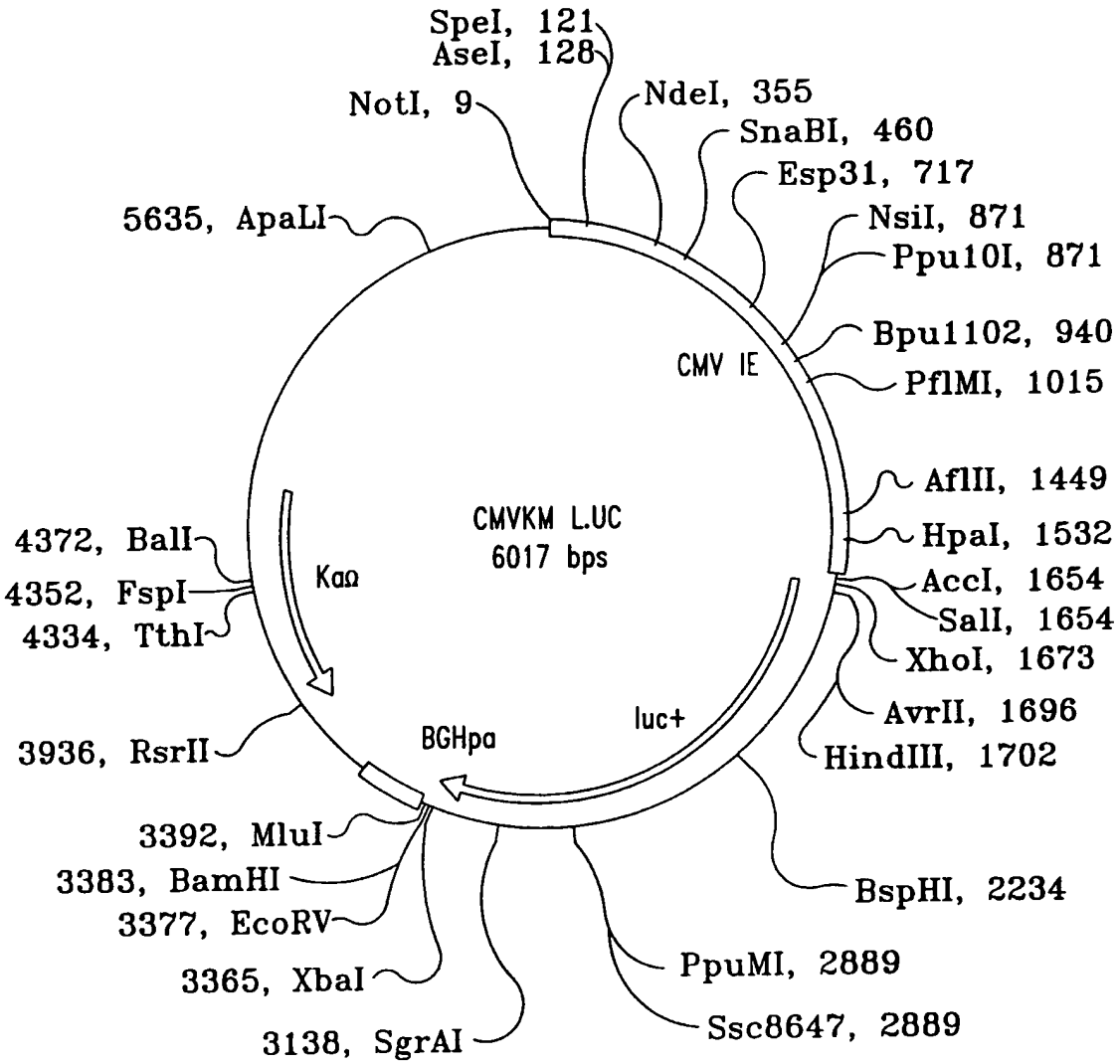

SpeI, 121
AseI, 128
NotI, 9
NdeI, 355
SnaBI, 460
Esp31, 717
NsiI, 871
Ppu10I, 871
5635, ApaLI
Bpu1102, 940
CMV IE
PflMI, 1015
AflII, 1449
CMVKM L.UC
6017 bps
HpaI, 1532
4372, BalI
4352, FspI
4334, TthI
Kαω
AccI, 1654
SalI, 1654
XhoI, 1673
AvrII, 1696
3936, RsrII
BGHpa
luc+
HindIII, 1702
3392, MluI
3383, BamHI
3377, EcoRV
BspHI, 2234
3365, XbaI
PpuMI, 2889
3138, SgrAI
Ssc8647, 2889

Fig. 5

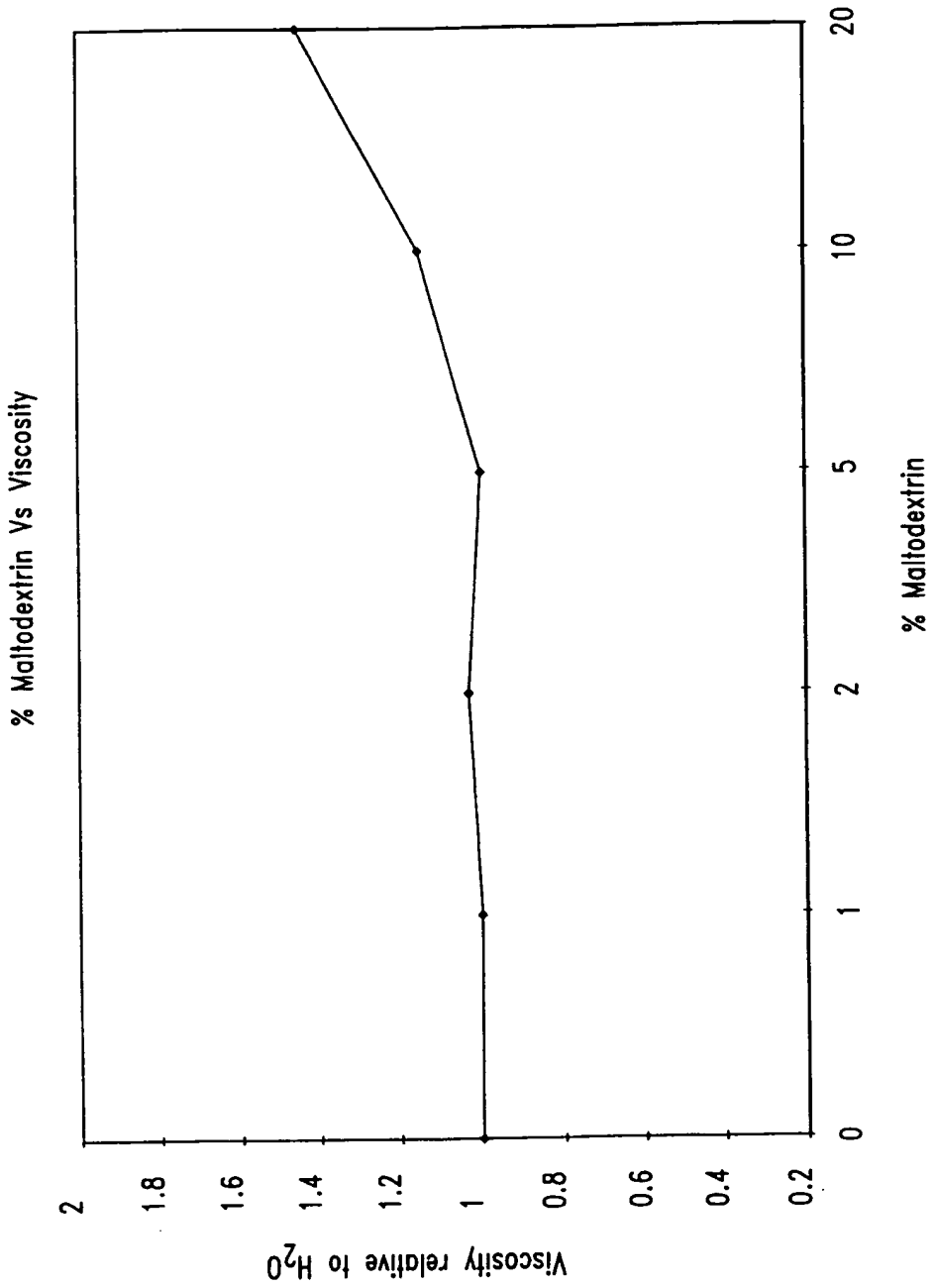

% Maltodextrin Vs Viscosity
Viscosity relative to H_2O
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
5
10
20
% Maltodextrin

Fig. 6

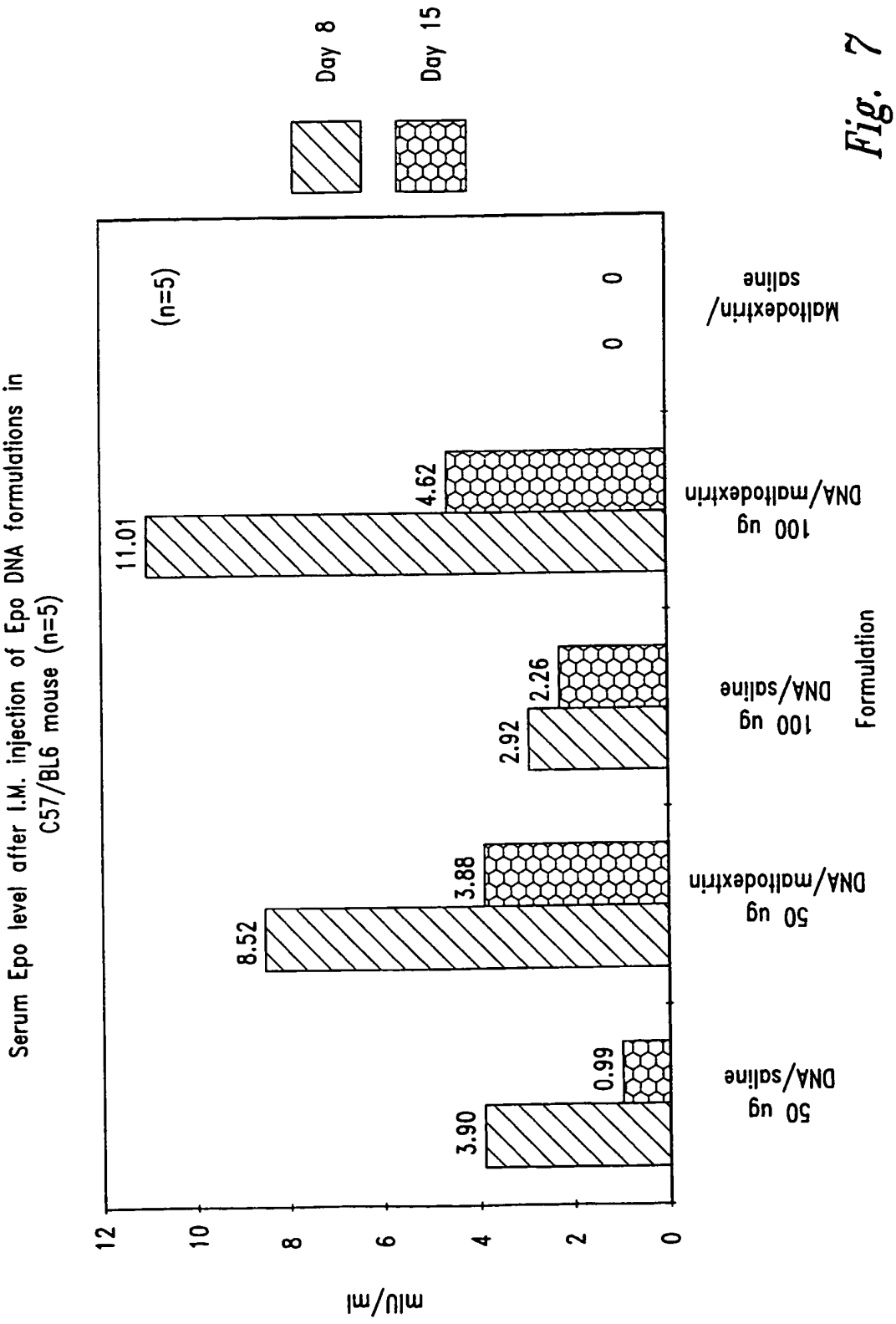

Serum Epo level after I.M. injection of Epo DNA formulations in C57/BL6 mouse (n=5)
mIU/ml
0
2
4
6
8
10
12
Day 8
Day 15
(n=5)
3.90
0.99
8.52
3.88
2.92
2.26
11.01
4.62
0
0
50 ug DNA/saline
50 ug DNA/maltodextrin
100 ug DNA/saline
100 ug DNA/maltodextrin
Maltodextrin/ saline
Formulation

Fig. 7

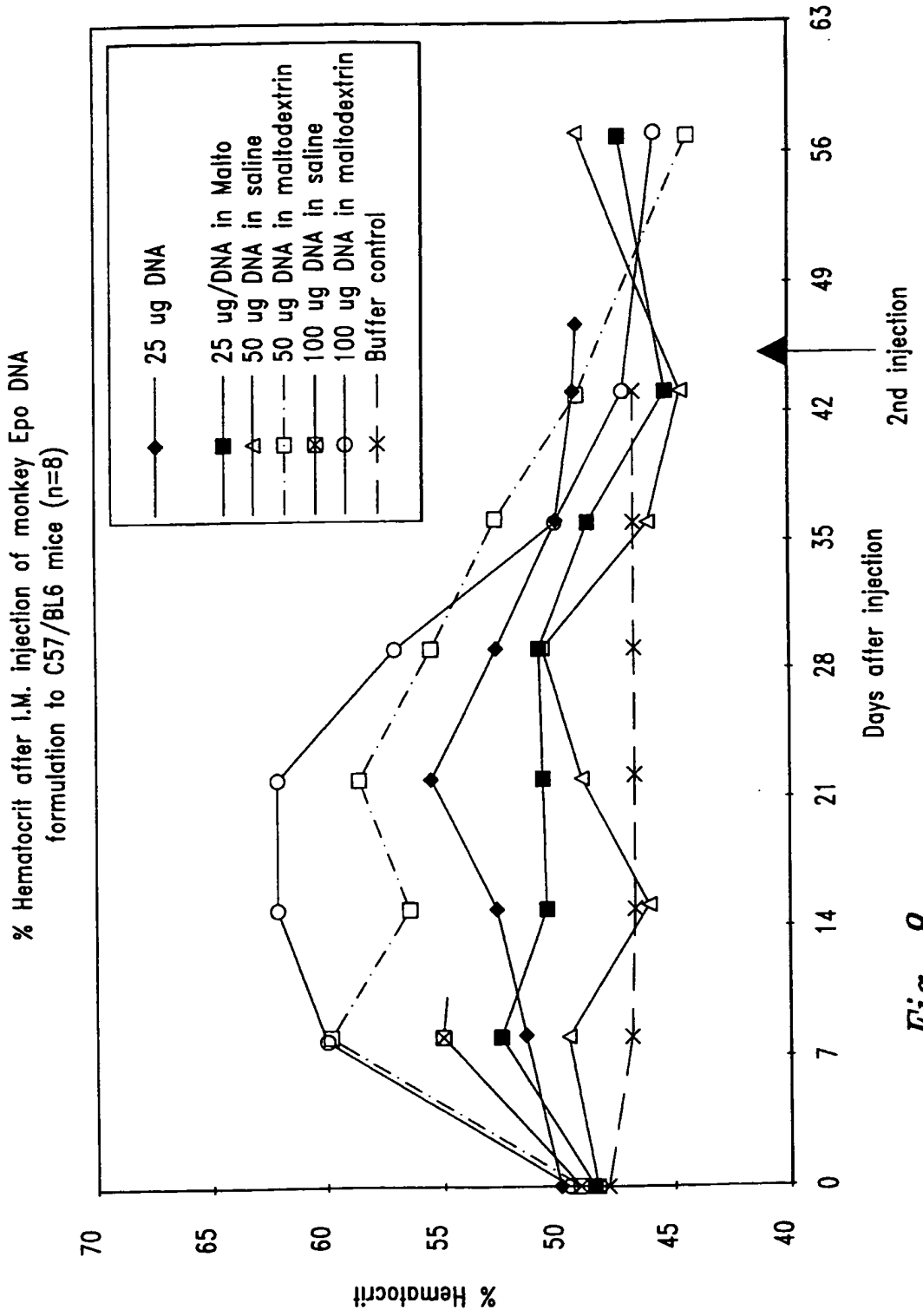

% Hematocrit after I.M. injection of monkey Epo DNA formulation to C57/BL6 mice (n=8)
25 ug DNA
25 ug/DNA in Malto
50 ug DNA in saline
50 ug DNA in maltodextrin
100 ug DNA in saline
100 ug DNA in maltodextrin
Buffer control
% Hematocrit
70
65
60
55
50
45
40
0
7
14
21
28
35
42
49
56
63
Days after injection
2nd injection

Fig. 8

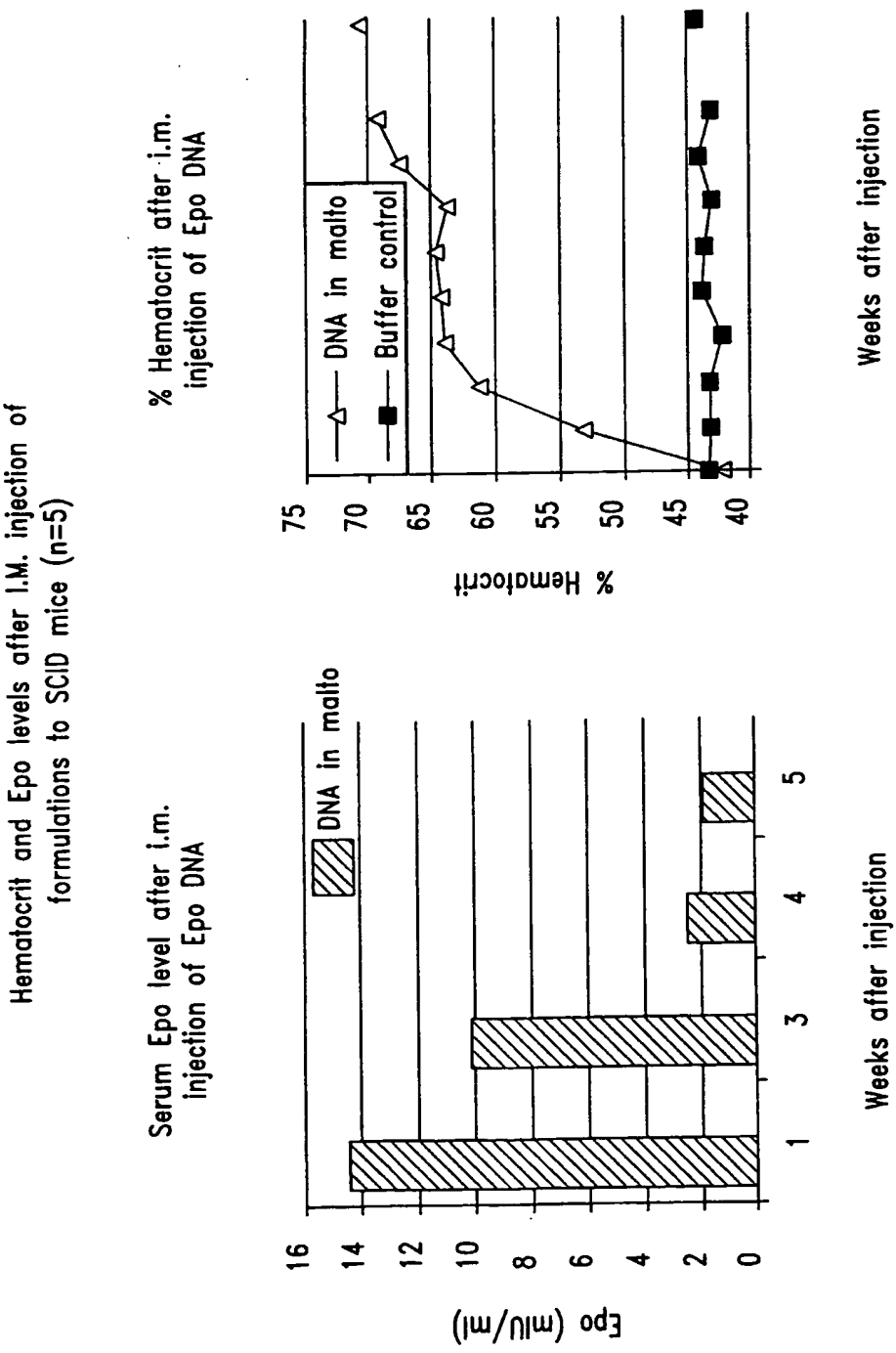

Hematocrit and Epo levels after I.M. injection of formulations to SCID mice (n=5)
Serum Epo level after i.m. injection of Epo DNA
DNA in malto
Epo (mIU/ml)
16
14
12
10
8
6
4
2
0
1
3
4
5
Weeks after injection
% Hematocrit after i.m. injection of Epo DNA
DNA in malto
Buffer control
% Hematocrit
75
70
65
60
55
50
45
40
Weeks after injection

Fig. 9A

Fig. 9B